Functional Genomics of Tick Thioester-Containing Proteins Reveal the Ancient Origin of the Complement System

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Key Words

Complement • Hemocyte • *Ixodes* • α_2 -Macroglobulin • Phagocytosis • RNA interference • Thioester-containing proteins • Tick

Abstract

Ticks are important ectoparasites and vectors of multiple human and animal diseases. The obligatory hemophagy of ticks provides a formidable route for parasite transmission from one host to another. Parasite survival inside the tick relies on the ability of a pathogen to escape or inhibit tick immune defenses, but the molecular interactions between the tick and its pathogens remain poorly understood. Here we report that tick genomes are unique in that they contain all known classes of the α_2 -macroglobulin family (α_2 M-F) proteins: α_2 -macroglobulin pan-protease inhibitors, C3 complement components, and insect thioester-containing and macroglobulin-related proteins. By using RNA interference-mediated gene silencing in the hard tick *Ixodes ricinus* we demonstrated the central role of a C3-like molecule in the phagocytosis of bacteria and revealed nonredundant functions for α_2 M-F proteins. Assessment of α_2 M-F functions in a single organism should significantly contribute to the general knowledge on the evolution and function of the com-

plement system. Importantly, understanding the tick immune mechanisms should provide new concepts for efficient transmission blocking of tick-borne diseases.

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Introduction

Ticks are blood-feeding ectoparasites (Chelicerata, Arachnida) and vectors of a variety of pathogens (viruses, bacteria, fungi and protozoa) that pose serious health and socioeconomical problems [1]. Ticks of the genus *Ixodes* are three-host species, where all stages (larva, nymph and adult female) feed on a different vertebrate host and contribute to the pathogen transmission [2]. The feeding is a slow process, taking from a few days (larvae and nymphs) to more than a week in adults. Before the adult female drops off the host, it consumes more than half a milliliter of blood, which accumulates in numerous gut diverticulae. The excess of ingested fluid is continuously excreted from the gut by the midgut cells into the hemolymph, re-

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absorbed by the salivary glands and secreted back into the host wound [2].

Resistance to starvation and desiccation, long-lasting feeding and intracellular type of blood digestion in ticks offer an exceptionally favorable environment for pathogen proliferation and transmission. For instance during feeding, *Borrelia* spirochetes, the causative agents of Lyme disease, rapidly multiply in the tick gut lumen. After having crossed the midgut cells by as yet unknown mechanisms, they disseminate in the hemolymph and eventually reach the salivary glands, the strategic site for vertebrate infection [3]. Transmission of an intracellular bacterium of the genus *Anaplasma* [4] or of the apicomplexan protozoa of the genera *Babesia* [5] and *Theileria* [6] begins with the initial infection of the midgut cells and proceeds to the invasion of other tick tissues, including the salivary glands. In all cases, invading pathogens have to overcome tick immune responses, which constitute a critical point of the tick-pathogen interface. In spite of the recent advances [7], our understanding of the tick immune system still remains fragmentary. Sequencing of the first chelicerate genome of the deer tick *Ixodes scapularis* [8] and feasibility of RNA interference (RNAi) [9, 10] offer a unique opportunity to decipher the components of the immune system in ticks and to investigate the interplay between ticks and pathogens at the molecular level.

Proteins of the α_2 -macroglobulin family (α_2 M-F) are key constituents of the innate immunity of vertebrate as well as invertebrate animals. This evolutionarily conserved family comprises (1) α_2 -macroglobulins (α_2 Ms), (2) C3/C4/C5 components of the complement system, and (3) thioester-containing proteins (TEPs) [11]. α_2 M pan-protease inhibitors are abundantly present in the plasma of a variety of metazoan organisms and are believed to contribute to the immune defenses by regulating immune responses and guarding the organisms against the proteolytic attacks of invading pathogens [12]. The complement system is activated in vertebrates by the lectin, classical or alternative pathways that lead to the proteolytic cleavage of the C3 molecule that opsonizes pathogens for phagocytosis or killing through a lytic pore formation [13]. Insect TEPs opsonize various classes of pathogens to promote their phagocytosis and killing [14, 15]. For example, TEP1 from the malaria mosquito *Anopheles gambiae* binds to and mediates killing of *Plasmodium* ookinetes in the mosquito midgut [16, 17].

Two α_2 M protease inhibitors have been previously characterized in two tick species – *Ornithodoros moubata* (TAM) [18, 19] and *Ixodes ricinus* (IrAM) [20]. These proteins are synthesized as large precursors and become

functional upon proteolytic cleavage of the highly variable segment located within the central part of the molecule [21]. The cleavage induces a rapid conformational change leading to the exposure of the reactive thioester (TE) bond and entrapping the proteases in a macromolecular cage. Interestingly, the RNAi-based functional study of IrAM demonstrated an important role of this molecule in the phagocytosis by hemocytes of the tick pathogen *Chrysobacterium indologenes* [20, 22].

Here, guided by the tick genome information available in VectorBase, we performed a functional analysis of the α_2 M-F in *I. ricinus*. We show that the tick genome encodes nine members representing all major groups of α_2 M-F. Until now, all groups of α_2 M-F proteins have never been observed in a single organism. RNAi silencing of the newly identified genes revealed their nonredundant roles in the phagocytosis of Gram-negative bacteria. These results provide new insights into the evolution of innate immunity and could shed light on the origin and function of the complement system in animals.

Materials and Methods

Biological Material

Adult females and males of *I. ricinus* were collected in Ceske Budejovice in the Czech Republic. For all experiments, 25 females were fed in the presence of the same number of males for 5 days (semi-engorged females) on laboratory guinea pigs. Experimental animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 137/2008.

Tissue Dissection and Isolation of RNA

Tick hemolymph was collected from the wound made by cutting the front leg and used for individual phagocytic assays or RNA isolation. Tissues (guts, salivary glands and ovaries) were dissected on a wax-filled Petri dish. Total RNA was isolated using NucleoSpin RNA II kit (Macherey-Nagel).

Database Search and Phylogenetic Analysis

Genes coding for *I. scapularis* TE proteins were identified in the VectorBase (<http://iscapularis.vectorbase.org>) using both the 0.5 and 1.1 versions of the genome. Phylogenetic analysis was done as described before [23]. For the sequence alignment, regions of about 550 amino acids spanning the central α_2 M-F domain (pfam00207) and TE-containing domain (cd02897) were used.

RT-PCR Profiling

Total RNA (0.5 μ g) was reverse-transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche). For RT-PCR, tick α_2 M-F sequences were amplified with gene-specific primers or primers derived from *I. scapularis* sequences (online suppl. table S1, text S1; for all online suppl. material, see www.karger.com/doi/10.1159/000328851). *Actin* (AJ889837) served as a loading control.

RNAi Silencing

DNA fragments of tick α_2 M-F genes (186–574 bp) were amplified with gene-specific primers or primers derived from *I. scapularis* sequences (online suppl. table S1, text S1). Primers contained *Apa*I and *Xba*I restriction sites for further cloning into the pII10 vector with two T7 promoters in reverse orientation, and the dsRNA synthesis was performed as described previously [14]. The dsRNA (0.5 μ l, 3 μ g/ μ l) was injected into the hemolymph of unfed female of *I. ricinus* using a micromanipulator (Narishige). Control ticks were injected with the same volume of dsGFP synthesized under the same conditions from linearized plasmid pII6 [14]. After 24 h of rest in a humid chamber, ticks were fed on guinea pigs. At least two independent RNAi experiments were performed for each *IrAM* knockdown. The efficiency of each dsRNA was tested by RT-PCR and/or Western blotting (data not shown).

Cultivation of Bacteria

C. indologenes was cultivated in LB medium containing ampicillin (50 μ g/ml). *Escherichia coli* (strain SBS 363) and *Staphylococcus aureus* were cultivated in LB medium without antibiotics. All cultivations were done in an orbital shaker at 37°C. The overnight cultures (2 ml) were diluted with 5 volumes of fresh LB medium, incubated for another 2 h to reach about 10^9 colony-forming units/ml, centrifuged at 3,500 g for 10 min, and finally washed 2 times in L15 medium supplemented with 10% bovine fetal serum (L15-BOFES). Spirochetes of *Borrelia burgdorferi* (strain CB 53) were cultivated in BSK-H Complete Medium (Sigma) as described before [20].

Phagocytic Assay and Methylamine Treatment

In vitro phagocytic assays were performed as described previously [20]. Briefly, hemolymph collected from about 25 semi-engorged females was mixed with L15-BOFES medium. For the methylamine treatment assay, L15-BOFES medium was pre-mixed with 0.2 M methylamine or 0.2 M L-glycine (mock control). Hemocytes (4×10^4) within the hemolymph were allowed to adhere to the surface of a round microscope cover slip for 1 h. Then, 100 μ l of the bacterial suspension in L15-BOFES was added and incubated for 1 h at 28°C. Finally, slides were fixed with 4% formaldehyde, washed in 0.3% PBS-Tween 20, and blocked with 1% BSA and 10% goat serum. The phagocytosis of bacteria was detected by indirect immunofluorescence using rabbit antibodies against bacteria [22]. For each experiment, the phagocytic index was determined for at least 5 individual slides by counting the number of hemocytes with engulfed bacteria for a total of 100 hemocytes in the microscopic field. Resulting numbers were related to the average phagocytic index obtained for the dsGFP control taken as 100% in the respective experiment and expressed as relative phagocytosis.

Results

The Genome of *I. scapularis* Contains Nine Members of α_2 M-F

To identify α_2 M-F members in ticks, we screened in silico the genome of *I. scapularis* and identified 9 genes coding for putative TEPs (IsAM1–9) (online suppl. table

S2). Exon-intron structures of *IsAM7* and *IsAM9* were predicted using the GenScan software (<http://genes.mit.edu/GENSCAN.html>) and confirmed by sequencing of the corresponding regions from *I. ricinus* cDNA (online suppl. text S1).

Phylogenetic analysis across the conserved α_2 -macroglobulin family domain (pfam00207) and TE-containing domain (cd02897) clustered the identified tick α_2 M-F proteins into 4 groups: (1) α_2 Ms (IsAM1, 2, 4); (2) C3/C4/C5 complement-like factors (IsAM5, 6, 7); (3) TEP (IsAM3), and (4) macroglobulin complement-related proteins (MCRs; IsAM8, 9) (fig. 1a). This classification was further supported by characteristic domain arrangements (fig. 1b). All tick α_2 M-F proteins, except for IsAM2 and IsAM8, possessed a conserved TE site. IsAM5, 6 and 7 contained a putative anaphylatoxin and a C-terminal netrin-like (NTR)/complement C345C domain characteristic for vertebrate C3/C4/C5 complement components. IsAM9 harbored a low-density lipoprotein receptor class A (LDLa) domain, which had been previously found in several insect TEPs. As the first protein with this domain was referred to as MCR [24], we called this group α_2 M-F MCR proteins. Guided by these data, we renamed the individual tick genes according to their group classification within the α_2 M-F as follows: *A₂M1*, 2°, 3 (*IsAM1*, 2, 4); *C3-1*, -2, -3 (*IsAM5*, 6, 7), *TEP* (*IsAM3*), and *MCR1*°, 2 (*IsAM8*, 9) (the circlets indicate proteins without conserved TE, see online suppl. table S1).

Structural Features of the Tick α_2 M-F Proteins

Based on the provisional genome sequence data, we made initial structural predictions for the tick α_2 M-F members. Posttranslational cleavage within the arginine-rich motif of the linker region next to the anaphylatoxin domain is a typical feature of vertebrate C3-complement molecules. The proteolysis splits the molecule into α - and β -chains, which are held together by the intramolecular disulfide bridges. We identified similar motifs in the protein sequences of two tick C3-like molecules: C3-2 and C3-3. Interestingly, similar lysine-arginine cleavage motifs were also found in *A₂M1*, *A₂M2*°, and TEP (online suppl. table S2; fig. 1b). The corresponding region of C3-1 was missing in the genome sequence and, therefore, could not be analyzed (online suppl. table S2; fig. 1b).

Another important feature of the α_2 M-F is the presence of a catalytic residue located approximately 115 amino acids downstream of the TE site. An amino acid in this position often determines binding specificity of the TE [25]. For instance, in vertebrate C3 and C4 complement proteins this position is occupied by a histidine,

Fig. 1. Phylogenetic tree and domain organization of selected α_2 M family proteins. *Ixodes scapularis* α_2 M-F proteins cluster into four α_2 M-F groups (α_2 M, C3/C4/C5 complement, TEP and MCR groups) and possess conserved domain architecture with other invertebrate or vertebrate α_2 M-F proteins. **a** Unrooted phylogenetic tree reconstructed using the neighbor joining (NJ) method based on alignment of about 550 amino acid sequences spanning the conserved α_2 -macroglobulin (pfam00207) and TE-containing (cd02897) domains using ClustalX. Alignment and sequence descriptions are provided as supporting information (online suppl. text S2). *Homo sapiens* (Hu), *Mus musculus* (Mo), *Cyprinus carpio* (Carp), *Ixodes ricinus* (Ir), *Ornithodoros moubata* (Om), *Limulus* sp. (Li), *Carcinoscorpius rotundicauda* (Cr), *Anopheles gambiae* (Ag) and *Drosophila melanogaster* (Dm) are shown. Sequences of *I. scapularis* are emphasized by ovals. The circlets indicate absence of TE bond. Numbers at the branches represent bootstrap support values calculated using the NJ criterion with 1,000 replicates. Scale bar = 0.1 substitutions per site. **b** The upper panel shows the domain organization of putative *I. scapularis* α_2 M-F proteins. The middle panel shows the conserved domain organization of A₂M/TEP proteins and additional domains of C3/C4/C5 complement and MCR proteins. Domain abbreviations and nomenclature are according to the NCBI-conserved domain database (<http://www.ncbi.nlm.nih.gov/cdd>). A₂M_N = MG2 (macroglobulin) domain of α_2 -macroglobulin; A₂M_N_2 = α_2 -macroglobulin family N-terminal region; ANATO = anaphylatoxin homologous domain; LDLa = low-density lipoprotein receptor class A domain; A₂M = α_2 -macroglobulin family; A₂M_comp = A-macroglobulin complement component; A₂M_recep = A-macroglobulin receptor; NTR_complement_C345C = NTR/C345C domain. *H. sapiens* (Hu), *A. gambiae* (Ag) and *D. melanogaster* (Dm) are shown. The lower panel shows domain organization of invertebrate or vertebrate representatives of each α_2 M-F group. Scissors indicate putative posttranslational cleavage sites. Dots indicate sequence gaps. Asterisks indicate putative TE sites. Lengths of sequences and positions of individual domains are in scale. Scale bar = 100 amino acids.

which catalyses binding of TE to the hydroxyl groups (for example, sugars on the microbial surface). Similarly, all three tick C3-related molecules (C3-1, C3-2, C3-3) possessed a histidine at this catalytic site. Substitution at this position by a less nucleophilic residue redirects the TE binding specificity towards the amino groups and leads to the formation of a γ -glytamyl- ϵ -lysyl bond, for instance between α_2 M and an entrapped protease [26]. In the tick A_2M3 , as in the majority of other α_2 M, this catalytic site is occupied by an asparagine. However, in A_2M1 and A_2M2° , the asparagine residue is replaced by a serine.

Gene Expression Profile of the Tick α_2 M-F

To examine transcriptional profiles of the newly identified genes, we performed tissue-specific RT-PCR using cDNAs prepared from hemocytes, gut, salivary glands and ovaries of semi-engorged *I. ricinus* females (fig. 2). Tick A_2 Ms and MCRs were the most abundantly expressed genes detected in all tested tissues, with the exception of A_2M2° , whose expression was not detected in the gut. In contrast, the C3-like and TEP genes displayed a tissue-specific pattern of expression, where C3-1, C3-2 and C3-3 were mainly transcribed in hemocytes and TEP transcripts were detected only in the salivary glands.

Proteins of the Tick α_2 M-F Are Required for an Efficient Phagocytosis of Bacteria

We next tested whether tick α_2 M-F proteins are involved in the phagocytosis of bacteria by the tick hemocytes. We first examined requirement of the active TE bond for phagocytosis. To this end, we compared phagocytic indexes of bacteria incubated with hemolymph before and after methylamine treatment. Methylamine readily reacts with TE bonds and inactivates all TEPs potentially present in the hemolymph. Hemolymph collected from semi-engorged ticks was pretreated with methylamine, incubated with bacteria, and phagocytosis was visualized by indirect immunofluorescence using bacteria-specific antibodies [22]. The phagocytic index was determined as a percentage of phagocytic hemocytes. Phagocytosis was tested for cultured live Gram-negative *C. indologenes*, *E. coli*, *B. burgdorferi* and Gram-positive *S. aureus*. The methylamine treatment caused a significant decrease in the phagocytosis of *C. indologenes* and *E. coli* (fig. 3a). In contrast, phagocytosis of *B. burgdorferi* and *S. aureus* was not affected, suggesting that their engulfment was independent on the α_2 M-F members containing active TE.

Fig. 2. Transcription profile of tick α_2 M family genes. RT-PCR was performed on cDNAs of tissues dissected from semi-engorged females of *I. ricinus*. Hemocytes (Hem), salivary glands (SG), ovaries (Ov) and gut (Gut) are shown. The same number of amplification cycles (30) was used for all genes. *Actin* served as a loading control.

The role of individual α_2 M-F in phagocytosis of *C. indologenes* and *E. coli* was further examined by RNAi-mediated gene silencing. Unfed adult females were injected with gene-specific dsRNAs and one day later fed on guinea pigs. For each gene, hemolymph from 25 semi-engorged ticks was pooled and incubated with bacteria. Ticks injected with *dsGFP* served as a negative control for dsRNA injection. The phagocytic index was determined as described above. The relative phagocytosis was expressed as a ratio of the phagocytic index in the experimental group to the phagocytic index in the *dsGFP* control in each respective experiment. In the *dsGFP* control experiments, 21.6% ($\pm 5\%$) of tick hemocytes phagocytosed *C. indologenes*. Phagocytosis of this bacterium was significantly decreased by silencing of A_2M1 and A_2M2° . However, the most profound effect, reaching almost the methylamine treatment levels, was observed upon C3-3 knockdown. Silencing of other α_2 M-F genes also affected phagocytosis, but the levels of reduction, except for the C3-1, were not statistically significant (fig. 3b). The phagocytic index for *E. coli* in the *dsGFP* control was in the average of 33.7% ($\pm 6\%$). Silencing of TEP and C3-3 significantly reduced the phagocytic index to the levels of methylamine treatment (fig. 3c). Our results demonstrate

Fig. 3. Phagocytosis of bacteria by tick hemocytes. Hemolymph collected and pooled from 25 semi-engorged females was incubated with indicated bacteria for 1 h. The phagocytic index was determined as a ratio of phagocytic versus nonphagocytic hemocytes. **a** Effect of methylamine pre-treatment of the hemolymph (ME; inhibition of TEPs) on phagocytosis of *C. indologenes* (*C.i.*), *B. burgdorferi* (*B.b.*), *E. coli* (*E.c.*), and *S. aureus* (*S.a.*). Gram-positive bacteria (G^+) and Gram-negative bacteria (G^-) are shown. Glycine was used as a mock control. **b** Effect of RNAi silencing of α_2 M-F genes on phagocytosis of *C. indologenes* by tick hemocytes. **c** Effect of RNAi silencing of α_2 M-F genes on phagocytosis of *E. coli* by tick hemocytes. Dashed line indicates the average level of phagocytosis after methylamine pretreatment. The numbers next to the error bars indicate the number of independent experiments (ME treatments or RNAi knockdowns) and the number of phagocytic indexes counts. * $p < 0.01$; ** $p < 0.001$ versus *GFP* controls.

involvement of 4 of 9 tick α_2 M-F proteins in phagocytosis of Gram-negative bacteria, and reveal their nonredundant roles in tick immunity.

Discussion

The ever-increasing data from genome sequencing projects offer novel insights into the evolution of protein families. Previously, α_2 Ms and C3 proteins have been found in such diverse organisms as vertebrates, hagfish, sea anemones and sea urchins, and were believed to represent two evolutionarily ancient branches of α_2 M-F [12, 27]. The common knowledge based predominantly on biochemical analyses ascribed functions of pan-protease inhibitors to α_2 Ms and of opsonins to C3 proteins. More

recently, it became clear that dipteran insects lack α_2 Ms and C3 molecules, and instead use a novel third branch of α_2 M-F proteins, called TEPs, to defend themselves against a wide range of pathogens [11]. Here, by a functional analysis of the tick genome, we demonstrate that ticks, which are chelicerate arthropods, possess members of all four α_2 M-F classes, suggesting that a full set of α_2 M-F proteins was present in the arthropod ancestor. During evolution, invertebrates must have lost some branches of the α_2 M-F family, with the most striking example of *C. elegans*, which has only one representative TEP in its genome. Is loss of the family members translated into the loss of particular functions, and can other members of the family functionally substitute for the missing proteins and perform their functions? In insects, the functions of TEPs, which are organized in distinct species-specific clusters,

seem to differ from one species to another. In fact, only one group of α_2 M-F proteins, the MCR proteins, seems to be conserved and represented by an orthologous member in all insect species sequenced so far. Ticks have two members of this group, and our phylogenetic analysis suggests that arthropod MCRs form an independent group within α_2 M-F. Our results show that tick α_2 M-F proteins form four phylogenetically, structurally and functionally distinct classes. Until now, members of all α_2 M-F groups have never been observed in a single organism. Therefore, ticks represent a formidable model to study the evolution of α_2 M-F in metazoan phyla.

We have previously reported that phagocytosis of the tick pathogen *C. indologenes* in *I. ricinus* was mediated by the activity of a bacterial secreted metalloprotease and the tick protease inhibitor A_2M1 [20]. Here we extend our analysis to demonstrate that knockdown of both A_2M1 and A_2M2° significantly decreases phagocytosis of *C. indologenes*. However, none of the tick α_2 M genes is required for phagocytosis of *E. coli*. These results suggest that in ticks α_2 Ms may have a dual function or may exploit their protease binding properties for enhancing phagocytosis of protease-secreting bacteria.

Among arthropods, a homolog of the central component C3 of the vertebrate complement system has been identified only in the horseshoe crab (Chelicerata) [28]. We identified three tick C3-like molecules (C3-1, C3-2, C3-3), which possess domains characteristic of the vertebrate C3, including the crucial anaphylatoxin and the C-terminal NTR/complement C345C domains. Our results now show that one of the C3 proteins (C3-3) is absolutely required for phagocytosis of Gram-negative *C. indologenes* and *E. coli*.

TEPs form expanded families in dipteran insects and comprise for example 6 members in *Drosophila* and 15 members in the mosquito *A. gambiae* [11]. The presence of a TEP-like molecule in the tick genome (TEP) suggests that arthropod TEPs have individualized early in animal evolution from the other members of the α_2 M-F.

MCR-like proteins have been previously described only in insects [24]. In addition to the typical α_2 M or TEP-like structures, MCRs display an LDLa domain. This domain is found in a number of vertebrate lipoprotein receptors, like LDL receptor or α_2 M receptor [29], but also in C9 complement component, involved in the formation of the membrane attack complex [13]. The exact function of this domain in the insect MCRs is unknown. Phylogenetic analysis clusters tick MCR1 and MCR2 $^\circ$ into one clade with insect MCRs and together they form a separate arthropod-specific group within the α_2 M-F.

Our results did not reveal any significant role for the tick MCRs in the phagocytosis of tested bacteria. A recent report in *D. melanogaster* suggested a role for MCR (TEP6) in the phagocytosis of yeast [24] by cultured cells, whereas another study demonstrated expression of MCR in the larval imaginal disks [30]. As loss-of-function MCR mutants were larval lethal, the authors proposed that this protein plays a key role in *Drosophila* development.

Although further studies should evaluate the role of bacterial proteases and tick α_2 M-F proteins in the phagocytosis of a larger number of bacterial species, the data obtained here suggest that ticks possess a complement-like pathway involving two independent branches, which converge on the C3-3 protein. Indeed, depletion of this protein caused the most massive decrease in phagocytosis of two bacterial species by tick hemocytes. It is tempting to propose that A_2 Ms and TEP have evolved to interact with different classes of pathogens. In this scenario, A_2 M will be targeted to protease-secreting bacteria, whereas TEP will interact with other Gram-negative bacteria. These initial interactions will lead to the activation of the central C3-3 component.

Finally, interactions of tick α_2 M-F proteins and other pathogens have not been addressed by this study. Mosquito TEP1, an ortholog of the tick TEP, regulates *Plasmodium* development by binding to the surface of ookinetes and initiating their lysis. Depletion of TEP1 in mosquitoes by RNAi dramatically impairs efficiency of *Plasmodium* killing [16, 17]. It would be of enormous interest to examine roles of the tick TEP in transmission of apicomplexan protozoa of the genera *Babesia* or *Theileria*, close relatives of *Plasmodium*. Tick-borne diseases caused by these parasites devastate livestock production worldwide and are also of medical importance as causative agents of human infections. Understanding the molecular mechanisms that shape efficient parasite killing could provide new concepts for blocking the transmission of babesiosis and theileriosis in the field.

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